Engineering an iCRISPR HIV Reporter Cell Line to Investigate Mechanisms of Extracellular Vesicle Sensing and HIV Latency

By

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Abstract

The goal of this project is to engineer a human immunodeficiency virus type 1 (HIV-1) reporter cell line with an inducible Cas9 cassette to pave the way for studies of how cells sense the presence of extracellular vesicles (EVs) during HIV-1 infection. Our previous studies showed that release of virus from HIV-infected cells, both productively and latently infected, was stimulated by removal of EVs from culture medium. Lipid synthesis pathways were upregulated under these same conditions. Cas9-equipped cellular models of HIV-1 infection and latency models would enable genetic screens of host factors involved in EV sensing. To this end, we achieved stable and inducible expression of Cas9 protein in the TZM-bl HIV-1 reporter cell line by transgenic insertion of M2rtTA and Cas9 cassettes into the AAVS1 locus through CRISPR-mediated homology-directed repair. After antibiotic selection, the success of cellular engineering was fully characterized at the DNA, protein, and functional levels. This cell line can now be used to identify cellular factors that are involved in EV sensing and various aspects of HIV pathogenesis of HIV.

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Preface and Acknowledgements

This thesis is the product of my Master's research that was initiated in September 2018 and finished in April 2019. The research was conducted in the Department of Molecular and Comparative Pathobiology at the Johns Hopkins University School of Medicine in Baltimore, MD. The entire project was conducted under the mentorship of Dr. Kenneth W. Witwer. I would like to express the utmost gratitude for my mentor Dr. Witwer for all of the guidance throughout my four years in his laboratory, and he witnessed my personal and professional growth as a scientist along the way. His dedication and passion for science also inspired me to pursue an M.D./Ph.D. as a future career, integrating scientific research with patient care.

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最后也是最重要的，我一切的成就都来自于我的父母。我十分感谢我父母用他们的努力和牺牲为我创造了来美国深造的机会。远隔重洋，跨越时区，经历 8 年，我希望能够用这篇毕业论文来回报他们为我所付出的一切。

Table of Contents

List of Tables

List of Figures

Chapter 1: Review:

Roles of Extracellular Vesicles (EVs) in HIV-1 Pathogenesis and Implications for HIV-1 Eradication

1.1 HIV-1 Infection and Latency

Acquired Immunodeficiency Syndrome (AIDS) still is one of the most daunting epidemics in human history. Following the initial diagnosis of the first AIDS patient in the United States[1], significant progress been made towards understanding the disease, and enormous efforts from both the scientific and medical communities have ended the status of AIDS as a death sentence.

Human Immunodeficiency Virus (HIV-1 or HIV) was identified as the infectious, causative agent of AIDS in 1983. Since then, efforts of the scientific and medical communities to identify and administer effective antiviral treatments have transformed HIV/AIDS from a "death sentence" to a manageable chronic condition. Nevertheless, to date, HIV-1 has claimed more than 35 million lives[2], and it continues to threaten global health with 1.8 million new infections diagnosed in 2017[2]. HIV has also created a substantial economic burden. In the United States alone, the aggregate cost of people living with HIV/AIDS was approximately $10.7 billion higher than the costs of those living without HIV/AIDS from 2002 to 2011[3].

HIV-1 belongs to the *Lentivirus* genus of the *Retroviridae* family and has two groups of isolates: HIV-1 and HIV-2[4]. Like other viruses in the *Lentivirus* family, HIV-1 has a chronic disease progression, with a long period of latency, persistent viral replication, and central nervous system (CNS) involvement[4]. The HIV-1 virion has a diameter of around 100 nm and is enclosed by a lipid bilayer membrane. Associated with the membrane are viral glycoproteins gp120 and gp41 and host membrane proteins, such as ICAM-1[4]. The HIV-1 genome consists of two single-stranded, positive-sense RNA molecules that contain nine genes which

encode for fifteen different gene products[4,5]. All of the structural proteins are encoded in the *gag* and *env* genes, while non-structural proteins such as *pol*, *tat*, and *rev* encode key effectors and modulators of HIV-1 replication[4].

HIV-1 has a multi-stage life cycle[6]. Virus attachment to the cell is initiated by the high-affinity binding of HIV-1 glycoprotein gp120 with cellular receptor CD4 and coreceptors CCR5 or CXCR4[5]. Following fusion of the viral envelope with the cell plasma membrane, the viral core enters the cytoplasm and the RNA genome is reverse transcribed into a double-stranded DNA by viral reverse transcriptase[6]. The DNA provirus is then transported into the cell nucleus and integrated into the host genome by viral integrase[6]. Once integrated and activated, the provirus gives rise to spliced and unspliced viral RNAs, which may be translated into proteins or retained as full-length genomic RNA for packaging into virions. Progeny virions then bud from cellular membranes. Virions mature during and after release through the action of viral proteases, producing infectious particles[6].

Specific stages of the virus life cycle are targeted by pharmacologic agents that are collectively known as Highly Active Antiretroviral Therapy (HAART) or simply antiretroviral therapy (ART). The first HIV-1 treatment was monotherapy with azidothymidine (AZT), a reverse transcriptase inhibitor that initially blocked viral replication and decreased mortality and AIDS-associated opportunistic infections[7]. However, drug resistance soon evolved because of the error-prone nature of the viral reverse transcriptase. This outcome necessitated development of combination antiretroviral therapies[8,9], which consist of a two- to four-drug regimen that stops viral replication while denying opportunities for evolution of resistance. Without active viral replication, the immune system experiences at least partial recovery of function[10,11].

Despite long-term control, HIV-1 remains incurable due to the presence of viral reservoirs that harbor quiescent yet replication-competent HIV-1 provirus. HIV-1 latency is enabled by multiple factors, including proviral integration into

the genome of resting CD4+ T lymphocytes, little or no viral gene expression, and viral sanctuary sites[12]. The best studied latent reservoir is memory CD4+ T-cells[13,14], but other cells including macrophages and microglia may also contribute to the viral reservoir[15,16]. Cellular reservoirse may also exisit in several anatomical reservoirs or sanctuaries, such as lymphoid tissues, the genital tract, the central CNS, and the lung[17–20]. Several molecular mechanisms have been uncovered to explain post-integration latency of HIV-1, including deleterious viral genetic mutation[21,22], transcriptional interference[23,24], chromatin epigenetic restrictions[25,26], and absence or presence of certain cellular factors[27,28]. The HIV-1 latent reservoir is extremely stable, with a mean half-life of around 44 months, such that eradication is unlikely to be achieved with ART alone[29].

Diverse alternative HIV-1 eradication strategies are under active investigation and are a major priority in the field. These eradication approaches include early or intensified antiretroviral regimens[30], transplantation of hematopoietic stem cell with anti-HIV moieties[31,32], anti-HIV-1 gene modifications[33–38], editing or excision of integrated HIV-1 provirus[39–41], and "shock-and-kill" strategies in which pharmacologic treatment reverses latency to allow for the elimination of infected cells by host immune responses or viral cytopathic effects[42,43]. Identifying a novel and combinatorial means to reverse HIV-1 latency remains as a priority. Recent results also suggest that extracellular vesicles (EVs) might be harnessed to reverse HIV-1 latency[44–46].

1.2 Extracellular Vesicles

All cells, from microbes to plants and animals[47–49], secrete membranous vesicles termed extracellular vesicles (EVs). EVs comprise a wide array of double-leaflet membrane extracellular particles, including exosomes and microvesicles[50], and range in diameter from 30 nm to well over one micron (large oncosomes)[51]. EV macromolecular composition tends to reflect, but is not necessarily identical to,

that of the cell of origin [52]. EVs have been isolated from most cells, as well as biological fluids [53,54].

The first report of EVs described these entities, derived from blood plasma via ultracentrifugation, have procoagulant properties by Chargaff and West[55], and the nature of these platelet-derived and lipid-containing particles was later examined by the Wolf[56]. However, more studies have highlighted the significance of EVs due to its capacity to transfer information to other cells in paracrine and endocrine fashion, thus modulating recipient cell functions[54]. In 1996, Raposo and colleagues reported these vesicles were antigen-presenting and were capable of inducing T cell responses[57]. A decade later, multiple publications sparked substantial interest in EVs by demonstrating that EVs contain RNA molecules, including mRNA[58] and microRNA (miRNA)[59]. The vital significance of EVs was then demonstrated by their capacity to serve as messengers for intercellular communication in multicellular organisms.

1.3 Shared Biogenesis Pathways between EV and HIV-1

Both enclosed by a membrane and carry macromolecules such as nucleic acids, proteins, and such, HIV-1 virions could be considered as a special type of EV, in particular when we delve into the biogenesis pathway of EVs and HIV-1. Different types of EVs tend to have different origins, and their biogenesis pathways should be considered separately. Exosomes are of endosomal origin; their initial formation begins with the inward budding of the endosomal limiting membrane to form intraluminal vesicles, thus defining multivesicular bodies (MVBs)[60,61]. This process of MVB formation involves two distinct pathways: the endosomal sorting complex required for transport (ESCRT)[45,62] and lipid domain-induced budding[60,63]. Upon MVB fusion with the plasma membrane, intraluminal vesicles are released as exosomes, possibly mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[64]. Considerably distinct from exosome biogenesis, microvesicles bud off from the plasma membrane

through protrusion and fission; the molecular machinery involved is still yet to be elucidated[60,65].

EV biogenesis machineries are used by both nonenveloped and enveloped viruses[66]. HIV-1 can rely on host cellular machinery for its own assembly into nascent virions[67]. HIV-1 virion assembly and scission rely on viral Gag protein, and the detachment mediated by Gag is dependent on host proteins TSG101 and ALIX. Both proteins are required for cellular budding into the endosomal compartment by recruiting the ESCRT complex[67–69]. Therefore, this ESCRT-mediated biogenesis pathway is common between EV and HIV-1 biogenesis. HIV-1 developed this strategy to divert this machinery to sites of virion assembly to aid for its release in a topologically conserved fashion[67].

1.4 Pro-viral Effects of EVs on HIV-1

Upon HIV-1 infection, EVs shed from infected cells may facilitate disease progression as "accomplices," through the transfer of host or viral components to uninfected cells while also evading host immune surveillance [70]. For example, Nef protein, an viral accessory protein that is expressed in the early stages of the viral life cycle[71], can stimulate its own exit by the release of EVs, and released Nef proteins can cause activation-induced cell death of resting peripheral blood lymphocytes[72]. The transfer of virus-encoded Nef protein can contribute to suppression of humoral immunity through impairing IgG2 and IgA antibody class switching[73]. Nef proteins are also capable of counteracting SERINC3/5, host restriction factors against retroviral infection, by restoring virion infectivity[74]. Nef proteins can also impair T cell activation by interfering with trafficking Luk and LAT to the immunological synapse[75]. EVs released from HIV-1 infected cells can also carry viral Env (gp120) protein[76], and the delivery of this protein to uninfected cells may facilitate HIV-1 infection indirectly through the inhibition of plasmacytoid dendritic cells (pDCs)[77] and the interaction with cellular integrin $\alpha_4\beta_7$[78].

EVs from infected cells and in patient blood can contain HIV-1 ncRNAs, such as trans-activating response (TAR) element RNA, which could make a naive target cell more susceptible to HIV-1 infection[79]. These TAR RNAs can also stimulate the production of pro-inflammatory cytokines IL-6 and TNFβ, which could exacerbate inflammatory responses in chronic HIV-1 infection[80]. Other reported virus-derived microRNAs (miRNAs), called vmiR88 and vmiR99, may also be present in EVs isolated from infected cell culture and HIV-1 positive patient blood[81]. These vmiRs could potentially lead to chronic immune activation, thus enhancing HIV-1 infection indirectly[81]. Intact HIV-1 virions could be transmitted in EVs from infected dendritic cells to CD4+ T cells without *de novo* infection[82].

Also, by differentially incorporating and excluding certain host immunoregulatory molecules such as CD86 and CD80, EVs derived from certain infected cell lines could compromise CD4+ T lymphocytes functions[83]. EVs derived from infected cells could transfer HIV-1 coreceptors CCR5 and CXCR4 to other cells that are typically resistant to HIV-1 infection, and ultimately confer or enhance HIV-1 infection in these cell types[84,85].

1.5 Antiviral Effects of EVs on HIV-1

EVs released from uninfected cells are also capable of inhibiting viral propagation via multiple routes. One study has illustrated that EVs secreted from CD8+ T cells display potent noncytotoxic antiretroviral activity by inhibiting integrated HIV-1 transcription[86]. EVs purified from biofluids of HIV-1 seronegative donors have also demonstrated potency in inhibiting cell-to-cell trans-infection. EVs isolated from breast milk have been shown to block dendritic cell (DC)-mediated viral transfer to CD+ T cells by binding to DC-SIGN[87]. Healthy donor semen also contains EVs that can block HIV-1 post-entry replication by inhibiting reverse transcriptase (RT) activity[88]. Semen-derived EVs also have anti-inflammatory properties, and these properties could be beneficial

to the host by reducing the extent of HIV-1-induced inflammation and cell activation[89,90].

Similarly, EVs can exert their modulatory effects by transferring multiple types of antiviral effector molecules to the recipient cells, thus blocking viral infection or restricting viral propagation. Various publications have verified the enrichment of a myriad of mRNAs that are classified as host restriction factors, such as APOBEC3 family members and BST-2/tetherin[88,91,92]. Anti-HIV-1 microRNAs (miRNAs), such as miR-28 and miR-223, have also been reported to be enriched in EVs[93,94].

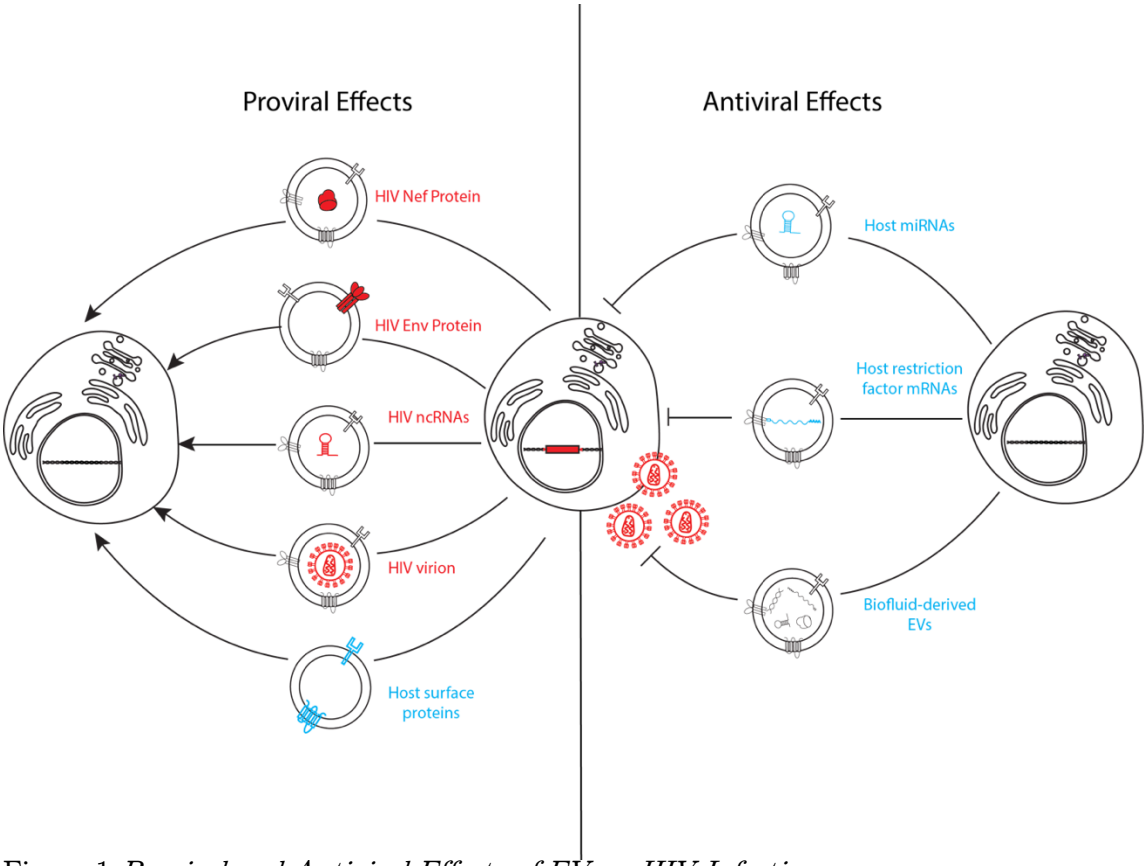

Figure 1 *Proviral and Antiviral Effects of EV on HIV Infection.*

EVs released from HIV infected cells can contain both viral and host factors to facilitate viral infection by priming uninfected cells. EVs derived from healthy cells and various biofluids have the potential of suppressing HIV infection by inhibiting HIV replication through the transfer of restriction factors.

1.6 Effect of EVs on HIV-1 Latency/Reactivation

EVs not only have broad implications in mediating acute HIV-1 infection, but various studies have also indicated that EVs could potentially serve as the effector of activating latently infected viral reservoirs to maintain a transient viremia during the course of the disease. Arenaccio and colleagues discovered that latent HIV-1 could be reactivated upon exposure to EVs released by infected cells[46]. This finding was validated in both cell lines and primary cell cultures. They also provided evidence that ADAM17 molecules loaded into these EVs could trigger the reactivation via the Nef-ADAM17-TNFα axis[46,95]. Interestingly, this finding was corroborated in a non-human primate animal model—rhesus macaques[96].

However, two years after the aforementioned paper was published, the Kashanchi lab found that EVs derived from uninfected cells can also reactivate HIV-1 basal transcription in latently infected cells by phosphorylation of RNA polymerase II on the HIV-1 promoter[45]. EVs isolated from healthy cells of various lineages were exposed to different cell lines chronically infected with wild-type viruses, such as ACH2 (lymphocytic cell line), U1 (monocytic line), and OM10.1 (premyeloid line) cells[45]. By mixing and matching donor EVs and recipient cell lines, Barclay et al. discovered that autologously sourced EVs are superior at activating HIV-1 latency, possibly due to lineage specificity between the cell types that the EVs were derived from and the recipient cell types[45].

Recent evidence from our lab has demonstrated an alternative possibility of reversing HIV-1 latency by withdrawing EVs from latently infected U1 and ACH2 cell lines[44]. Under EV-depleted conditions, these two cell lines each underwent viral reactivation to a certain degree, and further functional analyses indicated that alterations in cellular lipid production could be a potential underlying mechanism for this reactivation[44]. In the same study, we demonstrated that HIV-1 virions produced in the EV-depleted condition were more infectious, and cells cultured in this depleted condition were more susceptible to infection[44].

From these results, we concluded that EVs and other serum particles, which are commonly replete both *in vitro* and *in vivo,* could inhibit HIV-1 infection and production[44]. Together, these studies suggest that manipulating EVs in the cellular environment, or modulating cellular recognition of EVs, could be used to affect HIV latency.

Whether it is supplying EVs isolated from infected or uninfected cells or depleting serum EVs from the culture medium, and despite some contrasting conclusions, these data collectively emphasize that EVs have roles in HIV-1 latency. The necessity of fundamentally understanding the molecular interplay between EV homeostasis and HIV-1 latency maintenance in HIV-1 reservoirs calls for the identification of host factors that are associated with recognition of extracellular particles that contribute to the maintenance of HIV-1 latency.

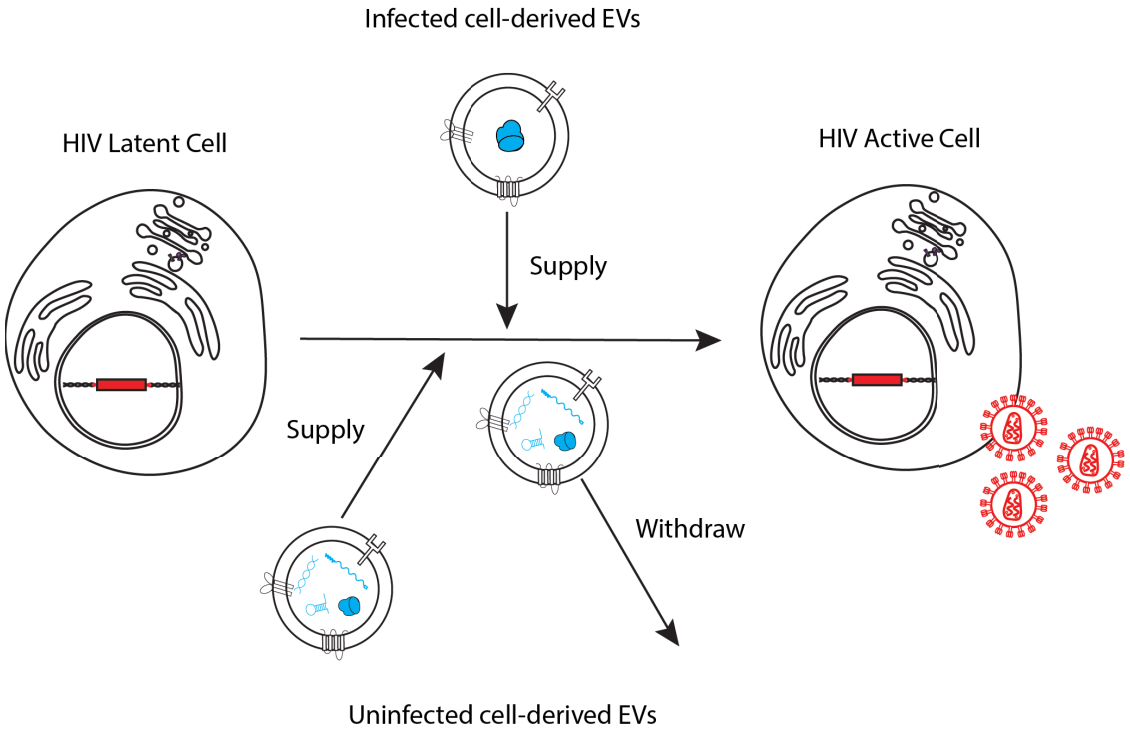

Figure 2 *Effects of EVs on HIV Latency*

Different routes that EVs can reactivate HIV latently infected cells: 1. Exposure of EVs derived from HIV-infected cells to HIV latent cells can activate HIV replication through the Nef-ADAM17-TNFα axis; 2. Exposure of EVs derived from uninfected cells to HIV latent cells; 3. Withdrawal of EVs derived from uninfected cells to HIV latent cells.

Chapter 2: Engineering an iCRISPR HIV Reporter Cell Line to Investigate Mechanisms of Extracellular Vesicle Sensing and HIV Latency

2.1 Introduction:

A cure for HIV-1 is a priority in today's scientific community despite effective combination antiretroviral therapy. Identifying novel approaches for latent reservoir eradication are currently in progress, and preliminary results from our lab and others suggest the possibility of harnessing extracellular vesicles (EVs) in novel HIV-1 eradication approaches[44]. EVs and HIV are closely related, and HIV virions can be even regarded as a specialized type of EVs subverted by the retroviral genomic reprogramming.

As EVs are commonly replete in cell culture medium due to the usage of bovine and human sera, it is crucial to minimize exogenous EVs from culture medium so as analyze endogenous EVs produced by the cells of interest. We have previously reported that depleting EVs from the culture medium of HIV infected cells affects various aspects of the retroviral life cycle[44]. Most importantly, EV depletion from culture media leads to increased HIV production by acutely infected cells and relaxes latency in both T-cell and monocyte-linage latency models *in vitro*[44]. By using gene arrays and lipidomics, we assessed cellular changes in response to EV depletion, and demonstrated that both the host RNA transcripts and the lipid products of specific lipid synthesis pathways (e.g., sterol biosynthesis) were significantly upregulated by cells exposed to EV-depleted conditions[44]. Hence, in the relative absence of serum lipid-containing particles such as EVs, lipids involved in both EV and virion biogenesis are upregulated (Figure 1A, B).

Therefore, we hypothesized that **enhanced lipid synthesis was a compensatory response to the perceived loss of the exogenous source of lipids transferred by EVs**. In turn, HIV virions are more likely to bud through membrane microdomains such as lipid rafts; hence this may explain our observation of HIV upregulation. There must **exist host mechanisms for monitoring incoming EVs or associated particles that likely involve cell membrane proteins or membrane trafficking components** (Figure 1C). By manipulating these host mechanisms, perhaps we can mimic the effects of EV depletion, to reserve HIV latency and ultimately eradicate HIV.

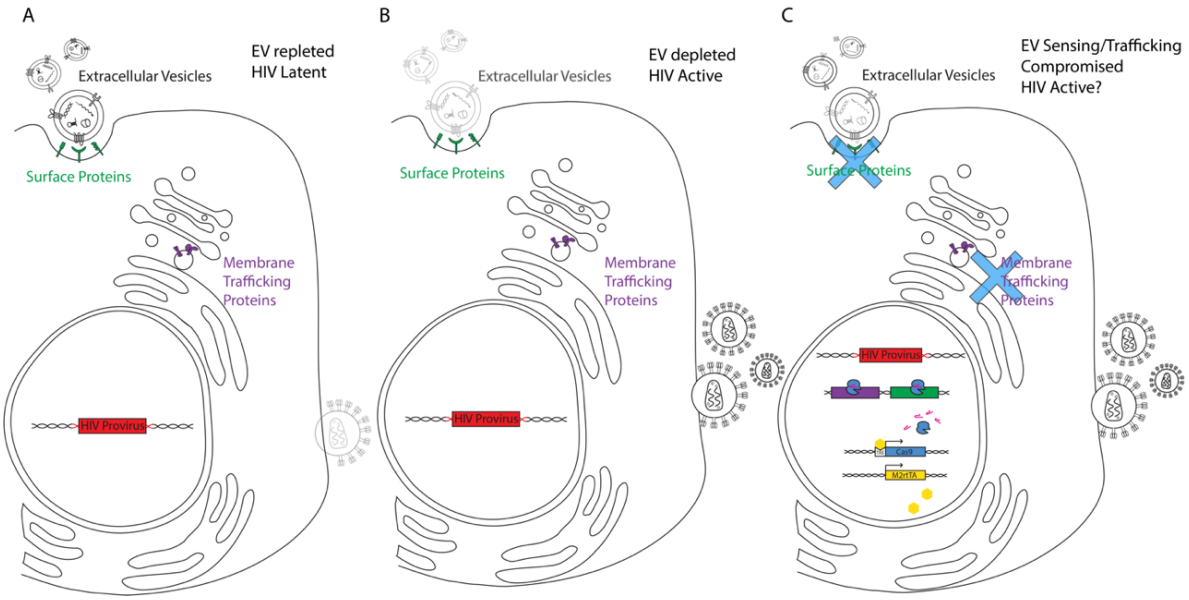

Figure 3. Visual illustration of project rationale.

A) When extracellular vesicles and other serum particles were present in cell culture medium, HIV latent cell lines remained latent, with minimal HIV-1 replication. **B)** When these particles were depleted from the cell culture, HIV latent cell lines became reactivated with a noticeable increase in HIV-1 production. **C)** We propose to mimic EV-depleted condition by knocking out EV sensing and trafficking machinery (blue crosses), and identify pathways which may potentially contribute to the maintenance of HIV latency.

Here, through CRISPR-mediated focused genetic screening, we propose to identify and target host factors associated with recognition of extracellular particles that could contribute to HIV-1 latency maintenance. A recent publication by González and colleagues has demonstrated an efficient and versatile CRISPR/Cas9 mediated genome editing system in hESCs[97,98]. As compared with the transient expression of genes that are mediated by plasmid delivery, stably integrated and inducible Cas9 expression exhibits numerous benefits as it allows a versatile yet precise expression of the invariable Cas9 protein in the CRISPR system. The variable gRNA component can be easily supplied by transfection due to its small size, allowing for rapid and efficient genetic screening. For better control of gene expression, González and colleagues used TALEN-mediated gene targeting into the AAVS1 locus[97,98], which has been a validated safe harbor for sustained transgene expression in human cells[99]. However, instead of TALEN-mediated gene targeting, we chose CRISPR-mediated gene targeting due to its high efficiency and technical convenience[100].

We established the iCRISPR platform through CRISPR-mediated targeting of inducible Cas9 expression cassettes into TZM-bl cell line for the initial screening of membrane trafficking and cell surface proteins. Successful transgenic insertion of Cas9 protein while maintaining the integrity of the parental cell line was comprehensively validated on DNA, protein, and functional levels. We have established a reliable engineering method for the inducible and stable expression of Cas9 *in vitro*, thus providing a versatile tool for HIV-related genetic screening.

2.2 Materials and Methods:

Cell culture:

TZM-bl cells(Cat. # 8129) and J-Lat full length cells (8.4) (Cat. # 9847) were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bl

was cultured in complete DMEM medium (D10) prepared with 10% heat-inactivated FBS (GE Healthcare Life Sciences, USA. Cat #: SH3039603), 1mM L-glutamine (Thermo Fisher, MA, USA; Cat #25030081), 1mg/mL Pen-Strep (Thermo Fisher, MA, USA; Cat #15140148), and 10mM HEPES (Thermo Fisher, MA, USA; Cat #15630080). J-Lat clones 8.4 was cutured in complete RPMI (R10) prepared with the same supplemental reagents as D10. Both cell lines were cultured in vented T-75 culture flasks (Corning Costar, PA, USA. Cat #: 430641U). Cells were incubated at 37flC in humidified 5% CO_2 environment. TZM-bl cell monolayer was split 1:10 at confluency by treatment with 1X 0.25% trypsin, 1 mM Ethylenediaminetetraacetic acid (EDTA) (Gibco, Canada. Cat #: 15400-054, Lot #: 2027369) as described[65]. J-Lat 8.4 cells were split 1:3 around a concentration of 1×10^6cells/mL.

Plasmids:

All three plasmids were obtained from Addgene. AAVS1 T2 CRIPR in pX330 plasmid was deposited by the Kanemaki lab (Addgene plasmid #72833). Puro-Cas9 donor plasmid was deposited by the Huangfu Lab (Addgene plasmid #58409). Neo-M2rtTA donor plasmid was deposited by the Jaenisch Lab (Addgene plasmid #60843).

Plasmid Isolation and Characterization:

Plasmid propagation:

Plasmids were obtained as bacterial stabs from Addgene. They were then individually plated on LB agar plates containing 100 μg/mL ampicillin (Quality Biological, MD, USA. Cat#: 340-108231; Lot#: 722888). *E. coli* containing Puro-Cas9 and M2rtTA donor plasmids were cultured overnight at 30flC (37flC for *E. coli* containing AAVS1 T2 CRIPR plasmid). After visible single colonies formed, liquid cultures were made by inoculating 2mL of LB (Quality Biological, MD,

USA. Cat#: 340-004101; Lot#: 722699) + 100 µg/mL ampicillin sodium salt (Sigma-Aldrich, MO, USA. Cat #: A9518-5G, Lot #: BCBG2945V) with a single colony from the respective LB agar plates. Liquid cultures were incubated at their appropriate temperatures (30flC for *E. coli* containing Puro-Cas9 and M2rtTA donor plasmids and 37flC for *E. coli* containing AAVS1 T2 CRIPR plasmid) overnight with rigorous shaking (300rpm). To obtain large volume for plasmid DNA maxiprep, 1mL of overnight liquid culture was added into 300 mL LB+100µg/mL ampicillin. Flasks were incubated with shaking at appropriate temperatures (30flC for *E. coli* containing Puro-Cas9 and M2rtTA donor plasmids and 37flC for *E. coli* containing AAVS1 T2 CRIPR plasmid) overnight.

Plasmid Maxiprep:

Transfection grade plasmid DNA were isolated using the EndoFreeff Plasmid Purification Kit (Qiagen, Hilden, Germany. Cat #: 12362. Lot#: 160029720) as per the manufacturer's protocol. Isolated DNA was eluted in 250 µL nuclease-free water (Qiagen, Hilden, Germany. Cat #: 1039480, Lot #: 160034794). DNA concentration was then determined using NanoDrop™ One/OneC Microvolume UV Spectrophotometer (ThermoFisher, DE, USA. Machine #:0094684).

Restriction Enzyme Digest:

One µg of each plasmid was combined with 5µL of 10X CutSmart Buffer (New England Biolabs, MA, USA. Cat #: B7204S, Lot #: 2861712) and 1 µL of each endonuclease (New England Biolabs, MA, USA) as indicated in Table 1. The final reaction mixture was completed with RNase-free water to a final volume of 50 µL.

Plasmid	Endonucleases Used
Puro-Cas9 Donor	AgeI HF(Cat #: R3552S. Lot #: 0041411), SpeI HF (Cat #: R3133S. Lot #: 0131702)
Neo-M2rtTA Donor	EcoRV HF (Cat #: R3195S. Lot #: 0051206), NheI HF (Cat #: R3131S. Lot #: 0021311)
AAVS1 T2 CRIPR in pX330	EcoRI HF (Cat #: R3101S. Lot #: 0131604), XbaI (Cat #: R0145S. Lot #: 0431708)

Table 1. *Restriction Enzymes Used for Digestion Reaction of All Three Plasmids.*

Reaction mixture was incubated for 1 hour at 37 ˚C on a Thermomixer (Eppendorf, Hamburg, Germany. Machine #: 53552K747628), after which the endonucleases were inactivated at 65 ˚C for 20 mins (SpeI was inactivated at 80 ˚C for 20 mins).

Gel Electrophoresis:

1% agarose was made by dissolving 1g of Ultrapure agarose (Invitrogen, CA, USA. Cat #: 16500-500, Lot #: 0000481896) in 100mL of 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) (Invitrogen, Vilnius, Lithuania. Cat #: AM9869, Lot #: 00534786); then the mixture was microwaved until clear. The molten agarose solution was then poured into the casting apparatus with a comb until it was cooled. The gel was then allowed to cool to room temperature and then placed into the gel box. 6µL of 6X DNA loading dye (ThermoFisher, MA, USA. Cat #: R0611, Lot #: 00606810) was added to each 10 µL of each DNA sample to be separated, along with 8µL of 1kb DNA ladder (ThermoFisher, Vilnius, Lithuania. Cat #: SM0313, Lot #: 00182322). Sufficient amount of 1X TAE was then poured into the gel box to fully immerse the gel. The comb was lifted and all DNA samples were loaded into the appropriate wells according to the plate map. Power supply was then connected to the gel box and the gel was run at 80V for 10 mins and then the power was increased to 100V for one hour.

After the gel was run, it was retrieved from the gel box and then stained in running buffer containing 0.5 μg/mL ethidium bromide (Amresco, Ohio, USA. Cat #: E406-5ML, Lot #: 3197C436) for 15 mins. The gel was then visualized in a GelDoc Imager (Biorad, CA, USA. Machine #: 0086277) using the appropriate tray. The gel was auto-exposed for 10 secs, optimized for intense bands.

Sanger Sequencing of iCRISPR Plasmids:

DNA samples were prepared according to Genewiz's protocol (Genewiz, Inc, NJ, USA). For each sequencing reaction, plasmid templates were diluted to around 100ng/μL and 10 μL was aliquoted into 8-strip PCR tubes (BioRad, CA, USA. Cat #:TLS0801, Lot #: BR00433874). Primers used for sequencing all 3 plasmids and their sequences are shown in Table 2:

Plasmid	Primer	Sequence (5'-3')
Puro-Cas9 Donor	T7 (universal)	TAATACGACTCACTATAGGG
	SV40pA-Reverse (Universal)	GAAATTTGTGATGCTATTGC
Neo-M2rtTA Donor	T7 (universal)	TAATACGACTCACTATAGGG
	pCAG-Forward (custom synthesized)	GCAACGTGCTGGTTATTGTG
AAVS1 T2 CRIPR in pX330	U6 (universal)	GACTATCATATGCTTACCGT
	BGH-Reverse (custom synthesized)	TAGAAGGCACAGTCGAGG

Table 2. *Sequences of Sanger Sequencing Primers for All Three Plasmids.*

A total amount of 25 pmol was used for each sequencing reaction and they were mixed with template at the facility. The sequencing reaction volume was 15 µL.

TZM-bl Transfection and Selection:

Lipofectamine 2000 Transfection:

TZMbI cells were seeded at 4×10^6cells per well into 12-well plates the day before transfection. TZMbI cells were transfected with 2 µg of plasmids in total in varying combinations using Lipofectamine 2000 (Invitrogen/Life Technologies, Carlsbad, CA Cat #: 11668-019 Lot #:1467572) diluted in OptiMEM Reduced Serum Medium (Gibco, Grand Island, NY Cat #: 31985-070 Lot #: 1762285). Controls included mock transfections and transfection of 2 µg of pmaxGFP plasmids obtained in Cell Line NucleofectorTM Kit C (Lonza, NJ, USA. Cat #: VCA-1004, Lot #: F-11982). Plates were incubated for 6 hours at 37°C. After incubation, successful transfection was confirmed by examining uptake of pmaxGFP plasmid with an Eclipse TE200 inverted microscope (Nikon Instruments, NY, USA. Machine #: 0072745). Transfection medium was removed. The plates were washed with PBS and refed with 2 mL fresh D10 medium.

Antibiotic Selection:

Transfected TZMbI cells were allowed to recover for 72 hours and they underwent antibiotic selection per each plasmid type. For cells transfected with both Puro-Cas9 donor plasmid and AAVS1 T2 CRIPR plasmid, selection was with 0.1 µg/mL of puromycin (Gibco, China. Cat #: A11138-03/ Lot #: 2022504) until visible single colonies were formed. For cells transfected with both Neo-M2rtTA donor plasmid and AAVS1 T2 CRIPR plasmid, selection was with 300 µg/mL of G418 (Corning, VA, USA. Cat #: 30-234-CR, Lot #: 30234406) until visible single colonies were formed. For cells transfected with all 3 plasmids,

selection was with 300 μg/mL of G418 and 0.1 μg/mL of puromycin. Freshly-made selection media was changed daily. As cells approached confluency, antibiotic concentrations were gradually increased to 400 μg/mL of G418 and 0.2 μg/mL of puromycin.

Transfection validation:

Correctly engineered iCRISPR TZM-bl lines were further validated by PCR and gel electrophoresis for correct genomic insertion of both Puro-Cas9 and Neo-M2rtTA cassettes, by Western Blot to assess inducible Cas9 expression, and by immunocytochemical analysis to probe for inducible Cas9 expression and localization pattern.

DNA Isolation

Genomic DNA from transfected cells was isolated using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany. Cat #: 51304, Lot #: 160010049) according to the manufacturer's protocol. Around 5×10^6 cells were used for each isolation. After elution with 100 μL RNase-free water, nucleic acid concentration was measured using a NanoDrop™ One/OneC Microvolume UV Spectrophotometer (Thermo Fisher Scientific, DE, USA). DNA isolates were stored at -20 ° C.

Polymerase Chain Reaction:

800ng of each DNA isolate was combined with 50 μL of Taq PCR master mix (Qiagen, Hilden, Germany. Cat #: 201443, Lot #: 160044355), and 2 μL of 10 μM forward and reverse primers in PCR tubes. Primers were designed using Primer-BLAST,[70] and synthetic oligos were manufactured by Integrated DNA Technologies, Inc. (IDT, IA, USA). Primer sequences are indicated in Table 3:

Gene	Primer	Sequence (5'-3')
Puro-Cas9	Forward	GGACAACCCCAAAGTACCCC
	Reverse	GGGCTTGTACTCGGTCATCT
Neo-M2rtTA	Forward	GGATTCGGGTCACCTCTCAC
	Reverse	GTGCCCAGTCATAGCCGAAT

Table 3. *Sequences of PCR Primers Used for Validation of Cassette Genomic Integration*

RNase-free water was added to the reaction mix to obtain a final volume of 100 uL. PCR reactions were carried out in a thermal cycler according to the following cycling program (Table 4):

Steps	Time	Temperature (˚C)
Initial denaturation	3 mins	94
3 Step cycling: Denaturing	45s	94
3 Step cycling: Annealing	45s	5 degrees below the melting temperature of primer set
3 Step cycling: Extension	1 min (Repeat cycling 35 times)	72
Final extension:	10 mins	72
Hold:	Overnight	4

Table 4. *Thermocycling Conditions Used for PCR Reactions.*

Gel Electrophoresis:

Gel electrophoresis was performed as previously described. 6µL of 6X DNA loading dye (ThermoFisher, MA, USA. Cat #: R0611, Lot #: 00606810) was

added to each 10 μL of PCR reaction mixture, with 8μL of 100bp DNA ladder (ThermoFisher, Vilnius, Lithuania. Cat #: SM0321, Lot #: 00714077).

PCR Purification:

PCR reaction products were cleaned up using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany. Cat #: 28104, Lot #: 154038972) according to the manufacturer's protocol. 50 μL of PCR reaction product was used for each PCR purification reaction. After elution with 100 μL Buffer EB, nucleic acid concentration was measured using a NanoDrop™ One/OneC Microvolume UV Spectrophotometer (ThermoFisher, DE, USA. Machine #:0094684). Purified PCR products were stored at -20 ° C.

Sanger Sequencing of PCR Products:

DNA samples were prepared according to Genewiz's protocol. For each sequencing reaction, purified PCR templates were diluted to around 1ng/μL and 10 μL was aliquoted into 8-strip PCR tubes. Forward primers synthesized for PCR reaction were used to identify Cas9 and M2rtTA gene integration and a total amount of 25 pmol was used for each sequencing reaction. The total sequencing reaction volume that was submitted was 15 μL.

iCRISPR TZM-bl Cas9 Expression Induction:

Four iCRISPR TZM-bl lines (Clone 7, 14, 25, and 26) and WT unengineered TZM-bl cells were seeded into 6 well plates with around 20% confluency. They were cultured for 2 days to reach 50% confluency. They were then treated with or without doxycycline (2μg/mL) (Sigma Aldrich, MO, USA. Cat #: D9891-5G, Lot #: BCBL3540V) for 2 days. Cells were then lysed with 200 μL 1:1 mixture of RIPA buffer (Cell Signaling Technology, Danvers, MA. Cat #: 9806S) and protease inhibitor (Santa Cruz Biotechnology, Dallas, TX. Cat #: sc29131) for 20 mins at room temperature. Lysates were spun down at 10,000 g for 2 mins to

remove other insoluble debris and were stored at -20 ° C for BCA Protein Assay and Western Blot Analysis.

BCA Protein Assay:

Protein concentrations of all doxycycline untreated and treated cell lysates were assayed using Pierce BCA Protein Assays kit (ThermoFisher, DE, USA. Cat #: 23225, Lot #: NI177989) according to the manufacturer's protocol. 25 µL neat and 10X diluted cell lysates were used in the assay. Protein concentrations were determined using an iMark microplate absorbance reader (BioRad, Hercules, CA. Machine #: 15563) with a 570 nm test wavelength and based on the provided standard.

Western Blot:

Western blot was used to detect the presence of Streptococcus pyogenes Cas9 proteins in 30 µg of total protein for each iCRISPR TZM-bl cell line. 75 ng of TrueCut™ Cas9 Protein v2 was used as positive control. All samples were brought to final volume of 24 µL using 1X DPBS. 8 µL of Laemmli 4X sample buffer (BioRad, CA, USA. Cat #:161-0747 Lot #: 64077737) supplemented with 10% 2-mercapatolmethanol (Biorad, CA, USA. Cat #: 1610710, Lot #: L004109A) was added to each sample. After 5 mins incubation at 95 ° C, 30 µL of each was loaded into a Criterion TGX 7.5% gel (BioRad, CA, USA. Cat #: 5678024, Lot #:64086792). The gel was electrophoresed by application of 100V for 100 mins. The proteins were then transferred to a PVDF membrane (BioRad, Hercules, CA. Cat #: 1620177. Lot #: 30379A12), which was blocked with 3% milk (BioRad, CA, USA. Cat #: 1706404. Lot #: 64047053) in PBS+0.05%Tween20 (PBST) (Sigma-Aldrich, St. Louis, MO Cat #: 274348 Lot #: MKBF5463V) for 1 hour. The membrane was subsequently incubated with mouse monoclonal anti-CRISPR-Cas9 [7A9-3A3] primary antibody (Abcam, MA, USA. Cat #: ab191468, Lot #:GR3242232-1), at a concentration of 0.1 µg/mL

overnight. After washing the membrane with PBST three times, it was incubated with a goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology, TX, USA. Cat #: sc-2005 Lot #: F2718) at a 1:5,000 dilution for 1 h. The membrane was then incubated with a 1:1 mixture of SuperSignal West Pico Stable Peroxide solution and Luminol Enhancer solution (Thermo Scientific, Rockford, IL Cat #: 34080 Lot #: SD246944) for 5 min. The membrane was visualized for 150s using Azure c500 Imaging System (Azure Biosystmes, CA, USA.Machine #: 0096507).

Immunocytochemistry:

iCRISPR and WT TZM-bl cells were cultured in 6-well plates containing sterile coverslips. After the same induction schedule as previously described, fixation in 1mL of freshly prepared 4% paraformaldehyde (Sigma Aldrich, MO, USA. Cat #: 6148-500G, Lot #: MKCB4217) in PBS was conducted for 20 mins and then quenched with 1mL of 10 mM glycine (Biorad, CA, USA. Cat #: 161-0718, Lot #: 64027676) in PBS. Cells were then washed 3X with ice-cold PBS. Immediately after washing, cells were permeabilized using 0.1% Triton X detergenet (Sigma Aldrich, MO, USA. Cat #:, Lot #:) in PBS for 10 mins at room temperature, washed with PBS, and then placed in blocking solution (PBS with 2% bovine serum albumin (Cell Signaling Techonology, MA, USA. Cat #: 9998S, Lot #: 20)) for 30 mins. Cells were incubated with mouse monoclonal anti-CRISPR-Cas9 antibody [7A9-3A3] (Abcam, MA, USA. Cat #: ab191468 Lot #:GR3242232-1) in 2% BSA-PBS (1:250 dilution) overnight. After washing with PBS, they were then incubated for one hour with FITC conjugated goat anti-mouse IgG F(ab')2 secondary antibody (Invitrogen, IL, USA. Cat #: 31543, Lot #: TJ2652971A) and washed again. Coverslips were mounted on frosted microscope slides (Thermo Scientific, China. Cat #: 2951-001, Lot #: 17348-629238) with 10 µL ProLong Gold antifade mountant with DAPI (Invitrogen, OR, USA. Cat #: P36931, Lot #: 2030225). Slides were then imaged with Nikon Eclipse 90i (Nikon, Japan. Machine ID: 0081647) and analyzed with ImageJ software.

TZM-bl Luciferase Assay:

Luciferase activities were assessed under both induced and uninduced conditions. iCRISPR TZM-bl clones and WT TZM-bl cells were induced as previously described in 96-well plates. 4 days prior to performing the luciferase assay, 15,000 cells of each cell type were seeded into each well. 24 hours after, cells on the induction plate would be exposed to doxycycline-containing medium. 48 hours later, cells on both the uninduced and induced plates were exposed to H9/Rf HIV-containing-media from infected cells overnight. The luciferase assay was performed according to the manufacturer's protocol using the Luciferase Assay System (Promega, WI, USA. Cat #E1500, Lot #: 0000309373) and was read on a Fluoroskan Ascent FL luminometer (Labsystems, USA. Machine #: 0063969).

LentiArray™ CRISPR Control Lentivirus Particles Transduction

As preparation for genetic screening using LentiArray™ CRISPR libraries, CRISPR positive control lentivirus particles (ThermoFisher, CA, USA. Cat#: A32069, Lot #:1989459) were used to determine the optimal transduction Multiplicity of Infection (MOI) for iCRISPR clones. These positive lentivirus particles target the human HPRT gene, which initiates the metabolism of the cytotoxic antitumor agent 6-Thioguanine (6-TG). Cells with HPRT gene knocked out would be resistant to 6-TG treatment. Transduction was performed as described per manufacturer's protocol, enhanced with 8 μg/mL of Polybrene™ Transfection Reagent (Millipore, Darmstadt, Germany. Cat #: TR-1003-G, Lot #:3168045). As the Puro-Cas9 cassette already confers iCRISPR TZM-bl cells with puromycin resistance, the puromycin selection phase was omitted as we only aim to demonstrate functional gene knock out. 4 days after transduction, fluorescent images were taken under the same setting using an Eclipse TE200 inverted microscope (Nikon Instruments, NY, USA. Machine #: 0072745). After 7 days, transduced iCRISPR cells were switched to 100 μL medium containing 5

μM, 10 μM, and 15 μM 6-thioguanine (6-TG) (Sigma Aldrich, MO, USA. Cat #: A4882-100MG, Lot #: SLBW6998) for 5 days. Viability of cells after 6-TG treatment was assessed using crystal violet staining. 50 μL 0.5% crystal violet solution (0.5g crystal violet powder (Sigma Aldrich, MO, USA. Cat #: C6158-50G, Lot #: MKBT4497V) dissolved in 80mL of distilled water and 20mL of methanol (Fisher Chemical, Venezuela. Cat #: A456-500, Lot #: 187936)) was added into each well and the plate was shaken at room temperature for 20 mins on a rocker at 20 oscillations per minute. Staining solution was then discarded and plates were washed under gentle running water four times. The plates were then dried overnight and cells were lysed using 200 μL methanol. Cell viability was determined using an iMark microplate absorbance reader (BioRad, Hercules, CA. Machine #: 15563) with a 570 nm test wavelength.

2.3 Results:

iCRISPR Platform for Inducible, Rapid, and Multiplexable Genome Editing in HIV Latency Cell Lines

The overall goal is to engineer inducible Cas9-expressing cell lines, firstly the TZM-bl line, that could help us identify cellular factors that are important for HIV replication. TZM-bl (previously called JC53BL-13) is an indicator cell line derived from a HeLa cell line clone that was engineered to express CD4, CCR5, and CXCR4 for HIV infectivity[66]. The cells also have integrated copies of the luciferase gene under transcriptional control of the HIV-1 long terminal repeat[101], so that the level of HIV infectivity and transcription can be quantified by luminescence upon supply of the luciferase substrate. This engineered iCRISPR TZM-bl cell line could then be used for identification of candidate host factors of interest for HIV infection.

To prepare an inducible Cas9-containing TZM-bl cell line for a genetic screen of HIV-related factors, we introduced two cassettes into TZM-bl HIV

reporter cell line via lipid-based transfection and CRISPR-mediated homology-directed repair (Figure 1A). One cassette contains a doxycycline-inducible Cas9 gene (Puro-Cas9 donor), and the other carries a constitutive reverse tetracycline transactivator (M2rtTA) gene (Neo-M2rtTA donor)[97]. After a stable iCRISPR cell line was established, Cas9 expression would be induced by doxycycline treatment and gRNA-containing lentiviral particles from our libraries of interest would be delivered for the genetic screens (Figure 1B).

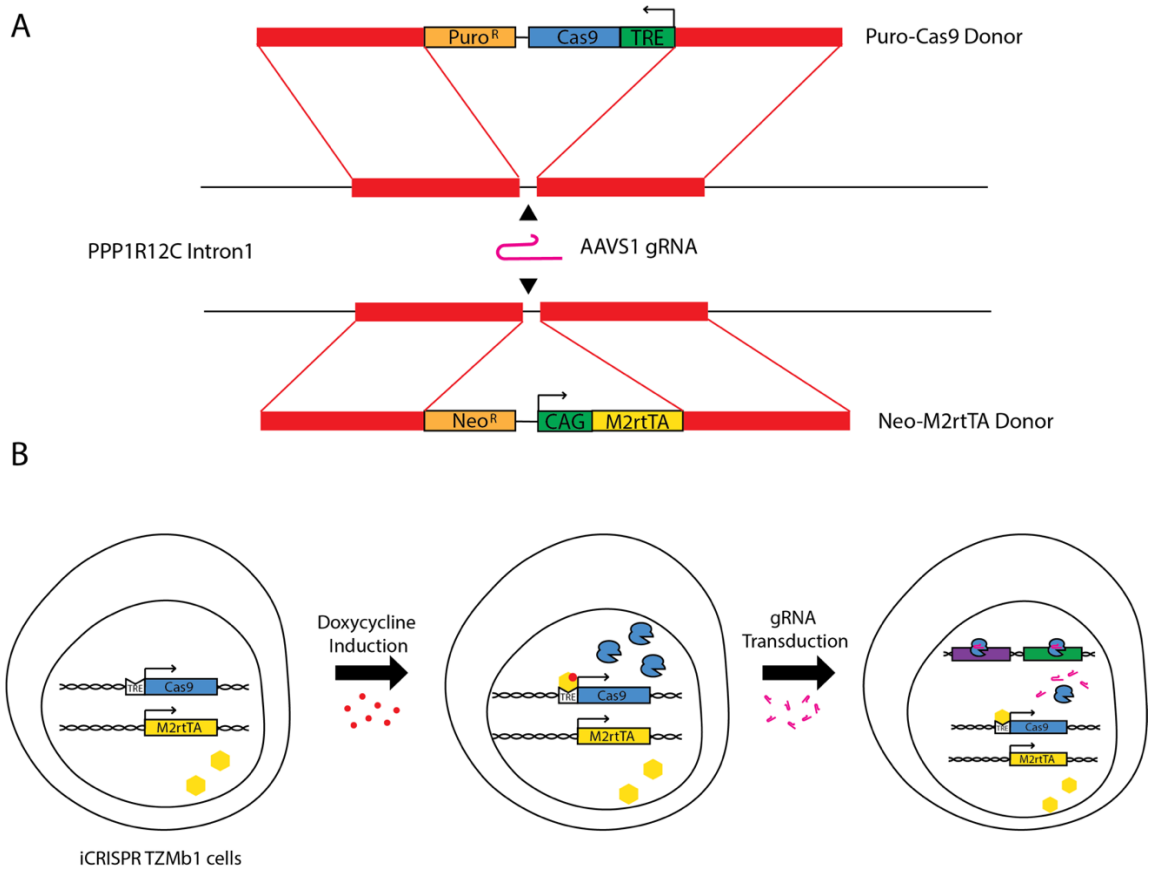

Figure 4. Workflow of iCRISPR TZM-bl Cellular Engineering and Genetic Screening

A) *Generation of iCas9 TZM-bl through CRISPR-mediated gene targeting at the AAVS1 locus.* Both Cas9 and M2rtTA cassettes will be inserted into PPP1R12C intron thorugh homology-dependent repair. Red segments indicate the homology regions between the donor plasmids and the TZM-bl genome. ***B)*** Schematic of iCRISPR-based genetic screen. Cas9 protein (blue) expression is induced with doxycycline (red dot). Doxycycline binds to M2rtTA (yellow hexagon), which in turns binds to TRE (white) to express Cas9. Cas9 protein binds with a gRNA molecule (pink)

to induce double stranded break to create gene knock-out for genetic screening purposes. TRE: tetracycline response element; CAG: constitutive synthetic promoter; M2rtTA: reverse tetracycline transactivator sequence and protein

Before transfecting the TZM-bl cell line, the three plasmids, one AAVS1-targeting Cas9-gRNA plasmid and the two donor plasmids described above obtained from Addgene, were thoroughly characterized. Their identities were verified using restriction enzyme digest and Sanger sequencing. Under various conditions, the sizes of the undigested, singly digested, and doubly digested plasmids were all presented at the correct size (Figure 2A). 2 primers were used for the Sanger sequencing of each plasmid and all sequences aligned well (Red regions) with the known sequences of the plasmids with minimal mismatch (white see-through regions on red arrows) (Figure 2B).

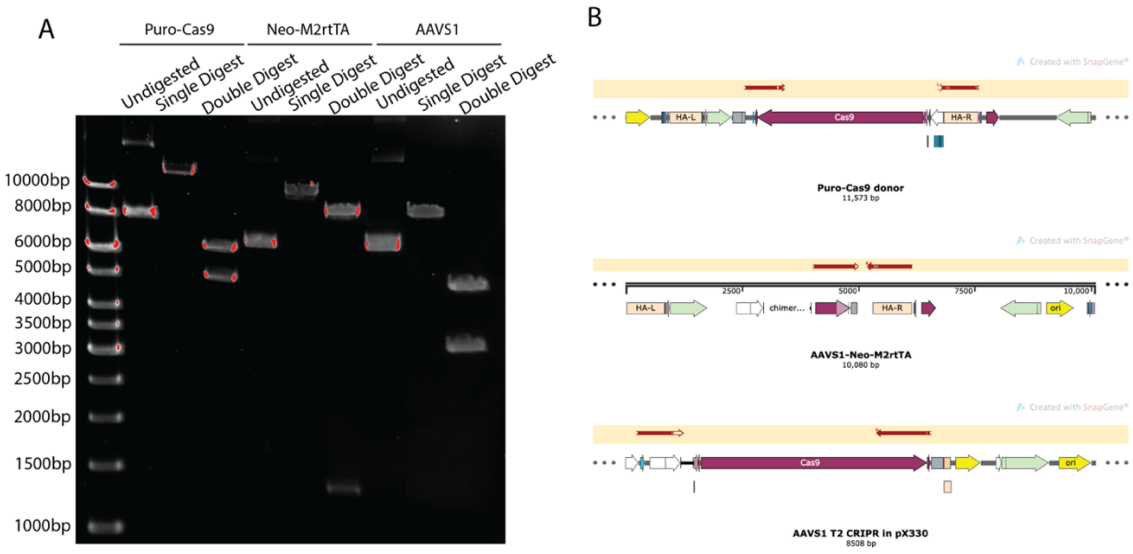

Figure 5. *iCRISPR Plasmids Characterization.*

A) *Restriction Digest Analysis of all three iCRISPR plasmids.* Lane 1: 10kb DNA ladder; Lane 2: undigested Cas9 plasmid; Lane 3: AgeI digested Cas9 plasmid; Lane 4: AgeI+SpeI digested Cas9 plasmid; Lane 5: undigested M2rtTA plasmid; Lane 6: EcoRV digested M2rtTA plasmid; Lane 7: EcoRV+NheI digested Cas9 plasmid; Lane 8: undigested AAVS1-CRISPR plasmid; Lane 9: EcoRI digested AAVS1-CRISPR plasmid; Lane 10: EcoRI+XbaI digested AAVS1-CRISPR plasmid. ***B)*** Sanger sequencing of three iCRISPR plasmids. Red arrows indicate homology between Sanger sequences with plasmid templates.

After comprehensive characterization of the plasmids, all three plasmids were introduced into TZM-bl cells using lipofectamine-based co-transfection. Transfected cells underwent one month of antibiotic selection to ensure a homogenous edited cell population. To comprehensively characterize the cell line, nuclear DNA was extracted from confluent clones, and successful genomic integration of both Cas9 and M2rtTA cassettes was confirmed in five (Clones 7, 14, 25, 26, 27) out of eleven clones using PCR and Gel Electrophoresis (Figure 3A). Western blot analysis also confirmed the induction of Cas9 expression upon doxycycline treatment in all four out of the five iCRISPR clones remained (Clones 7, 14, 25, 26) (Figure 3B). Clone 27 was not viable after successive passaging and selection using two antibiotics. Immunocytochemical analysis also revealed the presence of Cas9 protein in all clonal iCas9 lines, primarily localized in the cytosol without gRNA presence (Figure 3C). After all engineered iCRISPR TZM-bl clones were characterized at the DNA and protein level, the functionality of the iCRISPR clones was assessed to ascertain the intact reporter functionality of this cell line. Luciferase reporter activity of iCRISPR clonal lines was compared to WT TZM-bl cells under both uninduced and induced condition. Regardless of the production of Cas9 protein, all clonal lines besides clone 26 exhibited regular luciferase activity under the same infection condition (Figure 3D). Clone 26 was then excluded from future experiments since the elevated luciferase level would confound our findings in the future genetic screens.

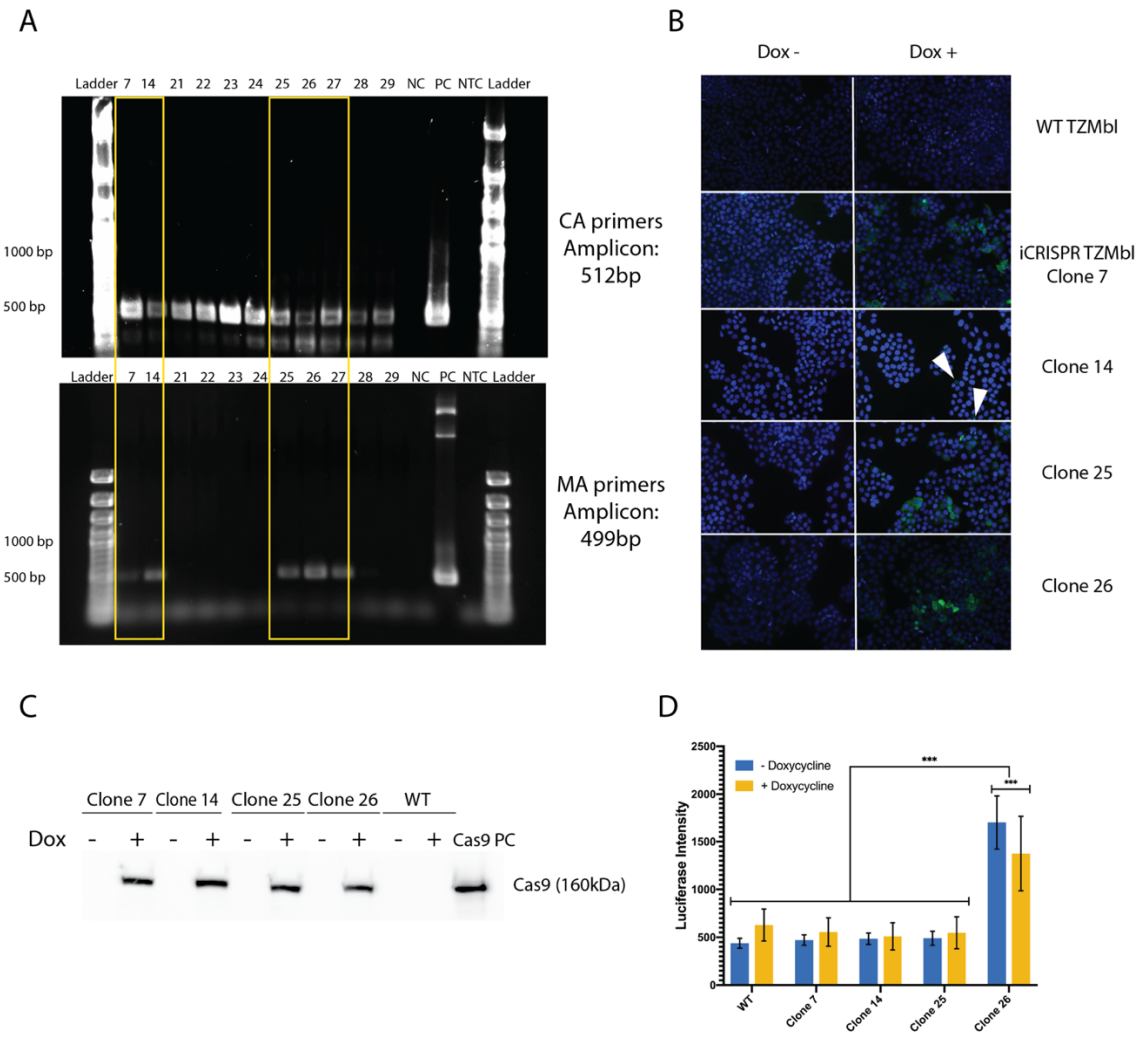

Figure 6. *Baseline Cellular Characterization of iCRISPR TZM-bl Clonal Lines*

A) *PCR analysis of iCRISPR TZM-bl clones.* 5 lanes in which the clones carrying desired cassette insertions in the AAVS1 locus are outlined in yellow. CA: Cas9/AAVS1 primers; MA: M2rtTA/AAVS1 primers. NC: negative control (WT TZM-bl DNA), PC: positive control (plasmids with known primer binding sites), NTC: No template control (H_2O) ***B)*** *Immunocytochemical analysis of Cas9 protein expression in all 4 iCRISPR TZM-bl clonal lines with or without doxycycline treatment.* WT TZM-bl cells were used as the negative control. White arrows in Clone 14 indicates faint green fluorescent signals compare to the negative control. **C)** Western blot analysis of Cas9 protein expression in 4 iCRISPR TZM-bl clonal lines with or without doxycycline treatment. WT TZM-bl cells were used as the negative control and commercially isolated Cas9 protein was used as positive control. ***D)*** *Luciferase reporter activity of in 4 iCRISPR TZM-bl clonal lines with or without doxycycline treatment.* WT TZM-bl cells were used as the negative control; *** $p<0.0001$ (2 way ANOVA using Sidak's multiple comparisons test and followed by Holme-Sidak correction for multiple tests).

The functionality of induced Cas9 protein was assessed in clone seven via 6-thioguanine resistance assay after the cells were transduced with human HPRT targeting lentiviruses. Optimal MOI was determined using various dilutions of lentiviral particles, and transduction efficiency was determined under a fluorescent microscope. MOIs of 1 and 0.1 were sufficient for transduction (Figure 4A left+right panels). iCRISPR clone7 demonstrated fully functional Cas9 proteins, as cells effectively have the HPRT gene knocked out were viable under 6-TG treatment compared with mock-transduced iCRISPR clonal cells (Figure 4B, C).

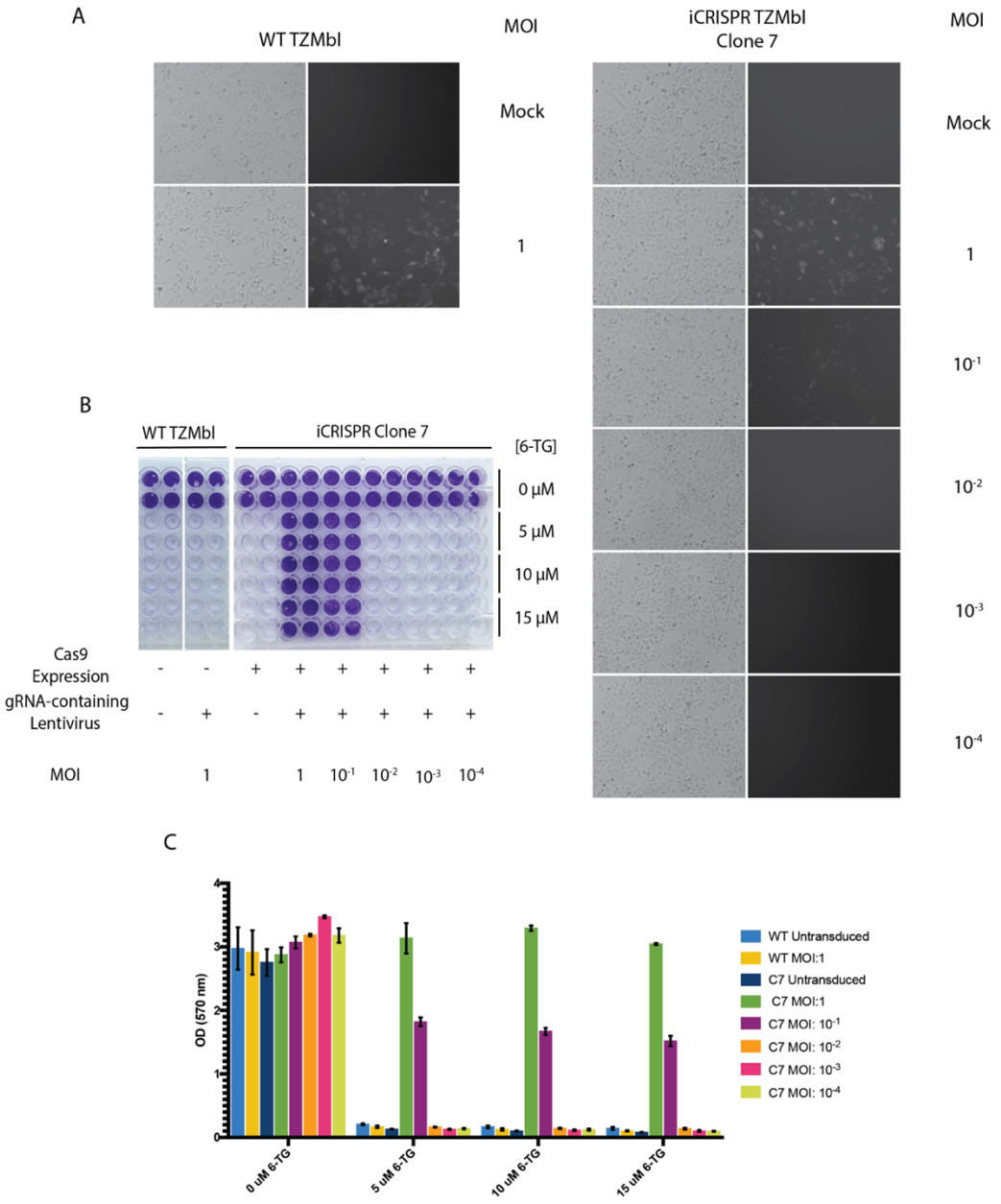

Figure 7. *Transduction Functional Characterization of iCRISPR TZM-bl Clonal Lines.*

A) *Light and Fluorescent microscopy images of transduced iCRISPR TZM-bl clonal lines.* Visible GFP signals were present in iCRISPR TZM-bl cells transduced with MOI of 1 and 10^{-1}. WT TZM-bl cells were transduced with the highest MOI and mock transduced iCRISPR cells were included as negative controls. ***B)*** *Pictures of crystal violet staining of transduced iCRISPR cells after 6-TG treatment.* **C)** Quantification of crystal violet staining assay using OD at 570nm. MOI of 1 and 10^{-1} were sufficient to confer 6-TG resistance at various concentrations to cells with HPRT knock-out.

2.4 Discussion:

Overall, iCRISPR TZM-bl cell lines have now been engineered with stable genomic integration of inducible Cas9. As an HIV indicator cell line, iCRISPR TZM-bl cell lines will now be used to screen host factors, including EV recognition molecules, that may affect susceptibility to HIV infection and capacity for HIV transcription.

By directly targeting the AAVS1 locus that is considered a safe harbor, the AAVS1 targeting approach that we took minimized the unpredictable effect of random transgene insertion through the traditional retroviral and lentiviral factors[102]. This directed insertion can be illustrated by the negative correlation between the amount of Puro-Cas9 amplicon and that of Neo-M2rtTA amplicon in the gel (Figure 3A), as there were limited AAVS1 loci in the genome available for insertion. One caveat that should be considered when targeting transgenes into AAVS1 locus is the fact that there exists a high degree of aneuploidy in immortalized cell lines. As demonstrated by Landry and colleagues, the HeLa cells, the parental line of TZM-bl, has a high degree of genomic segmentation and rearrangements[103]. As the transcriptomic profile is altered, the acceptability of using this cell line for genetic screening would be compromised.

As illustrated in the immunocytochemistry images (Figure 3B), the resultant clonal cells are mixed populations of engineered and non-engineered cells even after a long period of antibiotic selection. The heterogeneous nature of the clones with unpredictable genotype could benefit from a more efficient and uniform method of generating an iCRISPR system such as by combining CRISPR/Cas9-mediated genome editing with the Flp/FRT and Cre/LoxP system[104]. Not only intercellularly, but there also exist interclonal differences on translational and functional profiles. There is a stark difference in the percentage of Cas9 positively stained cells assessed by immunocytochemistry (Clone 14) and a difference in basal luciferase activities with or without doxycycline induction compared to the WT TZM-bl cells (Clone 26).

One thing to note is the importance of optimization in the initial phase of the experiments. Starting with plasmid transfection, the concentration of lipofectamine and nucleic acids that yielded a higher transfection efficiency was one μL Lipofectamine: one μg plasmid DNA, as the transfected cells reached confluence faster under antibiotic selection than cells under any other transfection conditions. In addition, each clone responded to various antibiotics differently, which required rigorous optimization of adequate concentrations of the antibiotics. To accomplish this, we implemented dose-response experiments to assess the antibiotic kill curve. These optimizations are critical to the success of cellular engineering.

A potential pitfall of this focused screen is that we may miss important factors or fail because gene combinations are needed to produce the effects, so we might not see any change in luciferase intensity in our genetic screen. Another potential pitfall is that there exist gene redundancies to complement the impact of gene knock out and we would likely to see no consequences either. These potential pitfalls could be circumvented by combining lentiviral particles that target multiple genes in a known pathway together and see the effect of certain established ontological pathways on the maintenance of HIV latency.

The high frequency of off-target activity could induce genetic changes at sites other than the on-site targets[105–108]. While full-scale genomic sequencing to rule out all off-target effects might be hard to achieve, we suggest that the repeated confirmation step is sufficient to dismiss a likely role for such activity. We could also verify that the results are indeed due to intended knock out by rescuing the phenotype with a plasmid encoding the WT gene that was knocked out.

Multiple HIV latent cell lines should be used in the next phase of the screening to ensure a validated result that has biological significance. In Sunshine and colleague's paper, they have discovered that four of the commonly used HIV

latency cellular models are significantly different from patient samples, both in terms of HIV integration sites and gene expression profiles[109].

This thesis provided evidence for the feasibility of the cellular engineering methodology that we employed and illustrated the versatility of this method in a diverse range of genetic screening projects. CRISPR-mediated knock-in using plasmid transfection worked well for this cell line and could potentially be used for any cell line of interest. For the engineered TZM-bl cells, we would likely deliver any gRNA of interest using a variety of methods to investigate the functionality of specific genes on the HIV life cycle.

Overall, one cell line has been engineered and fully characterized. The next step is to conduct an initial multiplex genetic screen with the commercial Membrane Trafficking Library to identify what are some potential cellular genes that are important in the HIV life cycle. The top candidates with the most substantial readout alterations would be validated again by individual knock-out experiments. After the initial screening in iCRISPR TZM-bl cell line, we would like to establish this iCRISPR platform in HIV latent monocytic U1 and lymphocytic ACH2 cell lines, to identify factors that could contribute to the sensing of EVs and the maintenance of HIV latency. The same lentivirus libraries would be used for genetic screens in these latency cell lines. Knock out of genes that produced the most considerable HIV latency reversal would be verified again using Bcl-2-transduced primary T-cell latency model that better recapitulate the quiescent state of restingCD4+ T cells *in vivo*[110]. Verified genes will be used for gene ontological analysis to elucidate the putative cellular mechanisms that could contribute to HIV latency maintenance.

This project will deliver novel insights into EV sensing by cells and how these interactions affect lipid metabolism and HIV latency. Druggable targets will be identified for adjunct HIV latency reversal approaches

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primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest*. 2009;119(11):3473-3486. doi:10.1172/JCI39199.

Curriculum Vitae

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EDUCATION

The Johns Hopkins University — **Baltimore, MD**
M.S., Molecular and Cellular Biology **GPA:4.00/4.00** — **May 2019**
B.S., Molecular and Cellular Biology **GPA:3.94/4.00** — **May 2018**
Minors:*Psychology, Spanish for the professions*
-Academic Honors: Dean's List, General & Departmental Honors, *Phi Beta Kappa, Beta Beta Beta*
-Danny Lee Award for Meritorious Research in Biology Science
-Provost Undergraduate Research Award

RESEARCH EXPERIENCE

Research Assistant — **Sept 2015 - Present**
Molecular and Comparative Pathobiology — Johns Hopkins School of Medicine
Principal Investigator: Dr. Kenneth W. Witwer
- Managed a project profiling the miRNA and extracellular vesicles (EVs) of the cervicovaginal compartment to identify potential biomarkers and restriction factors of HIV in simian models
- Proposed and carried out a research project of using the CRISPR system to decipher the cellular machinery involved in the complex interplay between EVs and HIV via a genetic screen
- Communicated my research internally and externally through symposium presentations, publications, and posters to both the Hopkins and international scientific communities

PrEP (Pre-Exposure Prophylaxis) Referral Program Research Coordinator — June 2018 - Present
Johns Hopkins Hospital Department of Emergency Medicine — Baltimore, MD
- Conceptualized and implemented the first ED-based HIV PrEP referral program to monitor and evaluate patients who are eligible for and interested in PrEP
- Formulated an comprehensive and streamlined online survey tool to screen and provide patients with PrEP referrals
- Educated patients about STD prevention and provided them with counseling upon diagnosis of HIV and Hepatitis C
- Coordinated with a team of physicians, case managers, and peer navigators by scheduling monthly meetings, reporting progress, implementing adjustments, and analyzing data for publications.

PUBLICATIONS & SKILLS

Publications
- **Zhao, Z.**, Muth, D. C., Mahairaki, V., Cheng, L., Witwer, K. W. (2017). Stem Cell Technologies in Neuroscience (Vol. 126).
- Muth, D. C., Powell, B. H., **Zhao, Z.**, Witwer, K. W. (2018). MiRNAs in platelet-poor blood plasma and purified RNA are highly stable: A confirmatory study. BMC Research Notes, 11(1), 15.
- Thery,C., Witwer, K. W., ...**Zhao, Z.** et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Jounrnal of Extracellular Vesicles, 7(1)

Presentations
- **Zhao Z.** Extracellular vesicle and miRNA profiling of the primate cervicovaginal compartment reveal possible anti-HIV defenses. Symposium Speaker at ISEV 2017 Annual Meeting; May, 2017; Toronto, Canada
- **Zhao Z.**Influence of Menstrual Cycle and Retroviral Infection on Primate Cervicov-

aginal Extracellular Vesicles and RNAs. Poster Presentation at 2016 CFAR Research Poster Session; Sept 2016; Baltimore, MD
- **Zhao Z.** Extracellular vesicle and miRNA profiling of the primate cervicovaginal compartment reveal possible anti-HIV defenses. Oral Presentation at CSEV 2018 Annual Meeting; Nov, 2017; Guangzhou, China
- **Zhao Z.** Jones, J., Sanders, R. et al. Emergency Department (ED)-Based HIV Pre-Exposure Prophylaxis (PrEP) Referral Program Using EDs as a Portal for PrEP Services. Poster Presentation at STI & HIV 2019 World Congress; July, 2019; Vancouver, Canada

Skills
- **Languages**: Madanrin (native), English (bilingual), Spanish (Intermediate)
- **Computer**: Microsoft, Adobe Illustrator, LaTex, Mendeley, Flowjo, Prism, PyMol, SnapGene
- **Research**: Cell culture, Nucleic acid isolation, Western Blot, Immunocytochemistry, ELIZA, Fluorescnet & Confocal Microscopy, Viability Assays, Nanoparticle Tracking Analysis, Transfection, Gel Electrophoresis, EV Isolation, PCR, RT-qPCR

TEACHING EXPERIENCE

Graduate Teaching Assistant Aug 2018 - Present
JHU Dept. of Biology Baltimore, MD
Course:
AS.020.151 & 153 General Biolgy & Laboratory (Dr. Rebacca Pearlman, Dr. Richard Shingles, Dr. Christov Roberson)
AS.020.374 & 377 Comparative Physiology & Laboratory (Dr. Anna Coppola)
- Coordinated with professors to reinforce critical content and the course objectives with students during classes and office hours
- Prepared teaching materials, evaluated students work to give constructive feedback, and patiently helped students one-on-one
- Organized cohesive lesson plans by incorporating instructional design and the students' needs

Peer-Led-Team Learning(PILOT) Leader Aug 2015- May 2018
JHU Academic Advising & Support Baltimore , MD
Courses Supported:
AS.030.101 Introductory Chemistry (Dr. S. Thyagarajan),
AS.180.102 Elements of Microeconomics (Dr. B. Hamilton),
AS.171.101 General Physics (Dr. M. Swartz)
- Led weekly 2-hour sessions to guide a group of 10-13 students in solving problem sets in Chemistry I and II, Microeconomics, and Physics I
- Worked with students one-on-one, led discussions and review sessions to clarify material.
- Managed 6 other PILOT leaders, curated weekly problems sets, and coordinated with the professors and teaching assistants to better prepare the students for their success in the course

Learning Den Tutor May 2018- December 2018
JHU Academic Advising & Support Baltimore , MD
Courses Supported: AS.200.141 Foundation of Brain, Behavior, & Cognition (Dr. L. Gorman)
- Held weekly review sessions for a small group of students to review the course content
- Coordinated with professors and teaching assistants to better cater the sessions to the needs of the students

CLINICAL EXPERIENCE

Generation Tomorrow HIV/HCV Counselor June 2016 - May 2018
Johns Hopkins Center for AIDS Research Baltimore, MD
- Certified in HIV/HCV testing, counseling, and education by Maryland Department

of Health and Mental Hygiene
- Conduct HIV/HCV testing in cohorts in Emergency Department in the Johns Hopkins Hospital and achieve successful Linkage to Care (LTC) upon detection
- Apply skills in community-based health agencies to respond to Baltimores HIV/HCV crises by doing field work on mobile clinics around the downtown area

2016 CFAR HIV/AIDS Scholar June 2016-Aug 2016
Johns Hopkins Center for AIDS Research Baltimore, MD
- Completed my research project Influence of Menstrual Cycle and Retroviral Infection on Primate Cervicovaginal Extracellular Vesicles and RNAs under mentorship of Dr. Kenneth Witwer and present it as CFAR research poster session in September, 2016.
- Participate in weekly lecture series where latest data on trends in HIV and HCV prevention, testing, and treatment are presented

Clinical Observer April 2017 - Present
Johns Hopkins Hospital Baltimore, MD
Wuxi People's Hospital Wuxi, Jiangsu, China
- Certified in HIV/HCV testing, counseling, and education by Maryland Department of Health and Mental Hygiene
- Conduct HIV/HCV testing in cohorts in Emergency Department in the Johns Hopkins Hospital and achieve successful Linkage to Care (LTC) upon detection
- Apply skills in community-based health agencies to respond to Baltimores HIV/HCV crises by doing field work on mobile clinics around the downtown area

COMMUNITY SERVICE EXPERIENCE

Safe Zone Program Facilitator Feb 2015 - Present
JHU Office of LGBTQ Life Baltimore, MD
- Actively promote an environment where the LGBTQA community flourish intellectually, socially, and emotionally everyone by educating students, staff, and faculty about the LGBTQA community
- Educated students, staff, and faculty by engaging them in 3-hour group discussions about LGBTQ issues, such as transgender identities and proper allyship.
- Provided the Hopkins community with respectful vocabulary and Hopkins-specific resources to promote a visible support network

Emergency Room Volunteer May 2016 - Present
Mercy Hospital Baltimore, MD
- Assist charge nurse with various duties, including making room visits to provide patients with warm blankets, drinks, and snacks, escorting patients during triage and discharge, as well as restocking patient rooms with medical supplies
- Identify patients' emotional needs and comforted or assisted them respectfully while communicating these needs to the care team in a sensitive manner
- Observe physicians and nurses as they examine and interview the patients, including walk-in patients and patients brought by the Baltimore Fire Department

Adult ESL Volunteer Teacher October 2016 - May 2018
Catholic Charities of Baltimore Esperanza Center Baltimore, MD
- Taught weekly beginner-level English classes to immigrants who were native Spanish and Mandarin speakers to instill confidence through language proficiency to promote integration in their local communities
- Designed individual lesson plans and tracked the students challenges and improvements to adjust their lesson plans accordingly

Volunteer/ Head of Family Sept 2014 - May 2017
Thread Volunteer Service Baltimore, MD
- Established supportive social networks for underperforming Baltimore high school students as a peer mentor
- Provided academic support in challenging courses for my tutee with fellow volunteers

and supervisors
- Organized after-school activities with Thread administration and other academic leads, including discussing current events, civil advocacy, and college/career planning

LEADERSHIP EXPERIENCE

Founder & President & Chief Editor June 2015 - Present
Blue Net China Multimedia Organization Baltimore, MD
- Founded the first student-run, annual publication that dedicates to showcase the influence of Hopkins in China and the American Chinese communities through our annual publication and social media
- Oversaw more than 120 interviews of Chinese alumni and faculty, which were published in our bilingual magazine
- Established a presence on three mainstream Chinese social media platformsWeChat, Zhihu, and Weiboreaching 5,000+ followers and 900,000+ views
- Organized a live-streamed virtual campus tour for Chinese students who could not visit the campus
- Held a student panel to help prospective students connect with appropriate resources at Hopkins
- Cooperate with the Office of Development and Alumni Relations and Johns Hopkins Technology Ventures to link Chinese resources with Johns Hopkins startups to efficiently move technologies to marketplace globally
- Engaged in collaborations with other cultural organizations by hosting events such as a U.S.-China Innovation Summit and a China-U.S. Health Tech Forum

International Ambassador President March 2017 - May 2018
Johns Hopkins University Office of International Services Baltimore, MD
- Led a team of ambassadors to provide incoming international students with essential academic and social support and fostered a tighter relationship between international students and the general student body at Hopkins
- Organized largest inter-school new international student orientation including nearly 1000 students, and a student career panel and summer welcome webinars
- Composed pre-arrival guides and OIS monthly newsletters